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PATENT



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Liew, C.C.

Examiner: Juliet C. Switzer.

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Titled: Method for the Detection of Gene Transcripts in Blood and Uses Thereof

Conf. No.: 8219

Commissioner for Patents
P.O. Box 1450
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DECLARATION OF- Paul R. Dobner, Ph.D, UNDER 37 C.F.R. §1.132

Sir:

I, Paul R. Dobner, Ph.D, hereby declare that:

1. I received a Ph.D. degree in Biology from Columbia Univ., NY, NY in 1981. I currently hold the position of Associate Professor in the Department of Molecular Genetics and Microbiology and the Program in Neuroscience at the University of Massachusetts Medical Center, Worcester, MA.
2. I perform basic research in neuroendocrine peptide function, including looking at differentially expressed genes in diseased states, as documented by my co-authorship of a recent journal article submitted to International Journal of Cancer, entitled Increased neurotensin receptor-1 (NTSR1) expression during progression of colonic adenocarcinoma. I have extensive experience in RNA isolation and detection, and have isolated RNA and DNA from blood. My c.v. is attached to this declaration.
3. I have read and understood the portion of the Office Action dated July 2, 2007 relating to Claims 17, 19-21, 23-24, 28-29, 31, 33-34, 38, 39, 41, 46, 49, 54, 55, and 56 as being anticipated by Ralph et al. (WO 98/24935 and 6190857).
4. The examiner states that the disclosure provided by Ralph et al. anticipates instant claims 17, 19-21, 23-24, 28-29, 31, 33-34, 38, 39, 41, 46, 49, 54, 55 and 56.
5. The examiner states Ralph et al. teach the responses secondary to disease states may be reflected in changing patterns of leukocyte mRNA levels that correlate with the presence of the disease state (Col. 5, lines 27-33).

6. The examiner states that Ralph et al teach the use of RT-PCR to identify two or more markers useful for diagnosing a disease, namely prostate or breast cancer, exemplifying this method for the detection of two transcripts referred to by Ralph et al. as UC331 and UC332, these sequences are RNA encoded by each of two genes (Examples 5.6.2 and following, Col. 98).
7. The examiner states that the genes are expressed in blood and non-blood tissues of subjects not having the disease (Col. 101, lines 41-47 and Col. 102, lines 5-10).
8. The examiner states that Ralph et al. teach using an oligonucleotide of predetermined sequence which are primers specific to the particular transcripts to detect a presence of RNA molecules (Col. 98, lines 17-19 and 26-27).
9. The examiner states that Ralph et al. detect a presence in samples from patients having prostate or breast cancer and from healthy volunteers (Col. 98, lines 5-6).
10. The examiner states that Ralph detect the presence of these RNA in DNA-free total RNA from peripheral blood (Col. 98, lines 5-6) and that DNA-free total RNA from peripheral blood is RNA of a blood samples which have not been fractionated into cell types, and likewise, it is obtained via the lysis of unfractionated cells.
11. The examiner states that Ralph et al. quantify the level of RNA encoded by the genes from both patients having disease and healthy patients, using relative quantitative RT-PCR (Col. 98, line 8).
12. The examiner states that Ralph et al. determine a difference between the levels of RNA in diseased and control samples, said difference identifying the gene as a marker of said disease (Col. 98, lines 32-37).
13. A. I disagree with the examiner that Ralph et al. describes what is set out in claims 17, 19-21, 23-24, 28-29, 31, 33-34, 38, 39, 41, 46, 49, 54, 55 and 56. These claims involve the detection of RNA in "blood samples that have not been fractionated into cell types". In contrast the examples from Ralph et al. referenced above involve the use of RNA prepared from the isolated mononuclear cell fraction of blood. In my expert opinion, "RNA of a blood samples which have not been fractionated into cell types" or obtained from "the lysis of unfractionated cells" is not described in Ralph et al. as stated by the examiner.
14. The examiner refers to the identification of two transcripts, referred to by Ralph et al. as UC331 and UC332, as being identified by RT-PCR (Example 5.6.2 and following, Col. 98). The RNA used for the identification of these two markers was isolated from peripheral blood mononuclear cells and not unfractionated blood. Ralph et al. state in 5.6.2 (in Example 6) that:

"1.5-5.0 µg of DNA-free total RNA from the peripheral blood of healthy volunteers or patients with either metastatic prostate or breast cancer were analyzed by relative quantitative RT-PCR as described in section 4.11.3 above."
15. This is the same text that is referred to by the examiner when she concludes that:

"Ralph detect the presence of these RNA in DNA-free total RNA from peripheral blood (Col. 98, lines 5-6) and that DNA-free total RNA from peripheral blood is RNA of a blood samples which have not been fractionated into cell types, and likewise, it is obtained via the lysis of unfractionated cells."

16. Following the written description of the procedure through carefully reveals that the RNA referred to is actually prepared from isolated mononuclear cells. In the first sentence of Example 6, Ralph et al. state that: "RNA fingerprinting was performed as described in Section 4.12 above" (Col. 95, lines 36-37). In the RNA fingerprinting method described in 4.12.2, Ralph et al. state that: "RNA was prepared as described in section 4.11.1 above" (Col. 70, line 8). Ralph et al. also make clear in Section 4.11.1 that the RNA prepared from nucleated circulating peripheral blood cells is used for both RNA fingerprinting and relative quantitative RT-PCR (Col. 67, lines 46).
17. The method for the preparation of RNA described in 4.11.1 refers back to Section 4.9.1 and explicitly states that: "RNA was prepared from nucleated circulating peripheral blood cells." Section 4.9.1 describes a detailed procedure used for the isolation of RNA that involves the collection of blood "into Vacutainer CPT tubes with ficoll gradients (Becton Dickinson and Company, Franking Lanes, N.J.)," and centrifugation "to separate the red blood cells from various types of nucleated cells, collectively referred to as the buffy coat, and from blood plasma" (Col. 62, lines 36-38) followed by the isolation of total cell RNA from the buffy coats by the RNA STAT-60 method (Tel-Test, Inc., Friendswood, Tex.).
18. The product information for Vacutainer CPT tubes (VDP40104) (Exhibit I) contains a detailed description as follows in this paragraph, of the composition of the separated cell fractions obtained using these tubes. The tubes contain an anticoagulant and a polyester gel to improve cell separation in addition to ficoll. Blood is collected in these tubes, the components are mixed, and the tubes are centrifuged resulting in the separation of different blood components and cell types due to the different densities of these components. The mononuclear cells and platelets migrate just above the polyester gel barrier while red blood cells and granulocytes pellet the bottom of the tube below the polyester gel due to the different densities of these different cell types. The mononuclear cells are readily obtained with a Pasteur pipette after removal of the plasma at the top of the tube. This isolated fraction is composed primarily of lymphocytes (B and T cells) and monocytes (destined to become macrophages). Most red blood cells and granulocytes are found in the cell pellet following centrifugation. Granulocytes are polymorphonuclear cells and include three distinct types, neutrophils, eosinophils, and basophils, which can be distinguished using different staining techniques, with neutrophils being the most numerous. Although these are the most numerous white blood cell types, they comprise only ~2% of the mononuclear cell fraction isolated using this method (BD Vacutainer CPT product information, VDP40104, Exhibit I).
19. Although blood fractionated in this way consists principally of mononuclear cells, mainly B and T lymphocytes and monocytes as just discussed, Ralph et al. refer to this fraction in various ways including "nucleated circulating peripheral blood cells" (Col. 67, lines 12-13), "the buffy coat" (Col. 62, line 38), and "peripheral blood leukocytes" (Col. 98, line 34). Thus, these three terms are used equivalently by Ralph et al.

20. The RNA used for relative quantitative RT-PCR can also be traced back to this method described in 4.9.1, since Ralph et al. refer back to the method described in Section 4.11.3. The RNA used in this procedure is prepared as described in Section 4.11.1, since after validation the RNA is used for both "RNA fingerprinting and relative quantitative RT-PCR" (Col. 67, line 46). Thus, in Example 6, Ralph et al. point back explicitly to the RNA isolation method described in Section 4.11.1 for the RNA fingerprinting method and point back implicitly to that same method for the relative quantitative RT-PCR procedure, making it clear that RNA was prepared from the isolated mononuclear fraction, and not "from blood samples that have not been fractionated into cell types" as stated by the examiner.
21. In Ralph et al. WO 98/24935, this method is explicitly included in the otherwise very similar Example 6 (p97, lines 13-26); thus, in my opinion there is no question that the RNA used in this Example was prepared from the isolated mononuclear cell fraction of peripheral blood samples. Although there is no specific linkage of this method to the relative quantitative RT-PCR method as there is in Ralph et al. 6,190,857, explicit references to the expression of the markers in peripheral blood leukocytes are included in the description of the relative quantitative RT-PCR results. For example, the last complete paragraph on p112 (WO 98/24935) opens with the statement "To independently verify that UC332 mRNA is more abundant in the peripheral blood leukocytes of patients with recurrent metastatic cancer as compared to the peripheral blood leukocytes of healthy volunteers, relative quantitative RT-PCR was performed using the same cDNAs and formats as were used to investigate the differential expression of UC331."
22. It should be noted that although Ralph et al. refer to "peripheral blood leukocytes", which is known in the art as a blanket term for all white blood cells, the detailed methods that they present in their working examples are for the isolation of mononuclear cells, including the T and B lymphocytes and monocytes, but largely excluding the more numerous granulocytes or polymorphonuclear cells. Thus, in my expert opinion, the term "peripheral blood leukocytes" as used in Ralph et al. clearly refers to the isolated mononuclear fraction.
23. This method also clearly applies to the use of oligonucleotides "of predetermined sequence which are primers specific to the particular transcripts to detect a presence of the RNA molecules (Col. 98, lines 17-19 and 26-27)" referred to by the examiner, since the paragraph describing the use of the oligonucleotides specific for UC331 and UC332 immediately follows the paragraph describing the method used for relative quantitative RT-PCR that is analyzed in the preceding paragraph.
24. The examiner also refers to Ralph et al. quantifying "the level of RNA encoded by genes from both patients having disease and healthy patients, using relative quantitative RT-PCR (Col. 98, line 8)." This specific reference is again in the initial description of the relative quantitative RT-PCR method (Col. 98, lines 5-11) that involves the use of RNA from isolated mononuclear cells as described above.
25. In addition, the examiner refers to Ralph et al. "determining a difference between the levels of RNA in diseased and control samples, said difference identifying the gene as a marker of said disease (Col. 98, lines 32-37)." The referenced text refers to the identification by RNA fingerprinting of "two cDNA fragments derived from mRNA species that had higher steady state abundances in the peripheral blood leukocytes of patients with recurrent metastatic prostate cancer as compared to a group of healthy volunteers" (Col. 98, lines 32-36). This example specifically makes reference to the "higher steady state abundance in peripheral blood leukocytes" indicating that RNA isolated from peripheral blood

leukocytes (i.e. isolated mononuclear fraction) was used for the RNA fingerprint analysis. The specific RNA fingerprinting method utilized in this Example is described in Section 4.12.2, which refers back to the RNA method described in Section 4.11.1. As illustrated above, this method describes the preparation of RNA from the isolated mononuclear cell fraction as described in more detail in Section 4.9.1.

26. These considerations make it absolutely clear that Ralph et al. use RNA prepared from the isolated mononuclear cell fraction throughout the examples referenced by the reviewer. Thus, Ralph et al. does not describe the use of unfractionated blood as set forth in claims 17, 19-21, 23-24, 28-29, 31, 33-34, 38, 39, 41, 46, 49, 54, 55 and 56.

B. All other relevant examples set forth in Ralph et al. involve the use of RNA prepared from mononuclear cells

27. In Example 2, Ralph et al. state that "RNA fingerprinting by PCR, primed with oligonucleotides of arbitrary sequence was performed on RNAs isolated from peripheral human blood" (Col. 79, lines 11-13) and subsequently in reference to the same experiment that "For this study, total cell RNA was isolated from buffy coat cells as described above" (Col. 79, lines 15-16). The only protocol that makes reference to the isolation of RNA from buffy coat cells is the one described in Section 4.9.1 (Col. 62, lines 33-48) where "nucleated" cells are fractionated in whole blood using Vacutainer CPT tubes with ficoll gradients and these nucleated cells are "collectively referred to as the buffy coat" (Col. 62, lines 37-38). Thus it is clear in my expert opinion that the RNA used in this experiment was prepared from isolated mononuclear cells in the buffy coat and that the authors use the phrases "RNAs isolated from "peripheral human blood" and "RNA isolated from buffy coat cells" interchangeably to reflect mononuclear cell RNA preparation.
28. In Example 3, Ralph et al. describe the "Identification of markers of metastatic prostate cancer by use of RNA fingerprinting by the pairwise sequential method" (Col. 79, lines 47-49). Three markers are identified by RNA fingerprinting using "the pairwise sequential method of McClelland et al (1994) as modified to use larger (17-25 mer) arbitrary oligonucleotides" (Col. 79, lines 51-52) and are referred to as band #'s 302, 321, and 325. Ralph et al refer to these bands as being either up or down regulated in the peripheral blood of prostate cancer patients (Col. 79, line 60-61, Col. 79, line 66-67). In fact throughout Example 3, Ralph et al. refer to these bands only in reference to peripheral blood; however, the authors use the phrase "peripheral blood" in reference to the mononuclear fraction of peripheral blood throughout the text and appear to use the phrase in this way here as well (see for example Section 4.9.1 where the authors refer to "nucleic acids isolated from the peripheral blood" in reference to RNA prepared from mononuclear cell fraction).
29. For instance, they refer to band #325 (corresponding to a previously identified species of IL-8 mRNA) as being "seven -fold more abundant in the peripheral blood of metastatic cancer patients" while an alternatively spliced form containing intron #3 is "up to seven-fold less abundant in the peripheral blood of metastatic prostate cancer patients" (Col. 80, lines 10-15). That Ralph et al. also use peripheral blood as a generic description for the isolated mononuclear cell fraction in this example can be inferred from a careful reading of Section 4.9.1. In my opinion, this Example can be clearly linked to the method described in Section 4.9.1, since this section makes specific reference to the RNA

fingerprinting method of McClelland et al. (1994) and Example 3 is the only one that utilizes this method (Col. 79, lines 50-53). Section 4.9.1 describes a method for the preparation of RNA from isolated mononuclear cells from peripheral blood of normal individuals and patients with metastatic prostate cancer. Notably, in Example 3, RNA fingerprinting is done using RNAs from only these two groups. Following the detailed description of the method for preparation of DNA-free RNA from isolated mononuclear cells, Ralph et al. describe dividing the RNA into three aliquots (Col. 62, lines 54-55). One aliquot was set aside for RT-PCR for standardization, a second aliquot was used for RNA fingerprinting using random hexamers for first strand cDNA synthesis and arbitrarily chosen oligonucleotides for subsequent PCR, and a third aliquot that "was pooled to make a pool of blood RNA from normal individuals and a pool of RNA from the blood of patients with metastatic prostate cancer" (Col. 62, lines 66-67, Col. 63, line 1). Ralph et al. then state in reference to these pools that: "The pools were fingerprinted using the sequential pairwise method of arbitrarily primed PCR fingerprinting of RNA (McClelland et al., 1994, Nucleic Acids Research 22, 4419-4431, incorporated herein by reference) with several changes" (Col. 63, lines 2-6). Example 3 is the only RNA fingerprinting experiment described in Ralph et al. that utilizes the method of McClelland et al. (1994) to identify markers (Example 5 makes reference to McClelland et al (1994) in the context of the IL-8 marker (Col. 89, lines 40-44), which was identified in Example 3). Thus, I conclude that Ralph et al. used the method described in 4.9.1 in Example 3 and therefore that the RNA was prepared from mononuclear cells isolated on ficoll gradients.

30. In Example 5, Ralph et al. present data concerning the use of relative quantitative RT-PCR to analyze IL-8 mRNA expression and immunological detection to analyze IL-8 protein expression. The title of this section indicates that: "Interleukin 8 (IL-8) mRNA and protein is up regulated in peripheral blood leukocytes of patients with metastatic prostate and breast cancer" (Col. 89, lines 29-31). However, in the description of the relative quantitative RT-PCR results in Section 5.5.2.1, Ralph et al. state that: "three pools of peripheral blood cDNA were examined" (Col. 91, lines 10-11). Further in the same section, they refer to their "failure to detect a PCR product in the peripheral blood of healthy volunteers" and "an abundant product of the expected size cDNA" as being "detected in peripheral blood RNAs of patients with metastatic breast or prostate cancer" (Col. 91, lines 33-37). In these experiments they find "that the mRNA for IL-8 is about 15 fold more abundant in the peripheral blood of patients with either metastatic prostate or breast cancer compared to normal individuals" (Col. 91, lines 48-51). They also analyze cDNA prepared from individuals and find that "compared to healthy controls, IL-8 mRNA was more abundant in the peripheral blood of all twenty metastatic cancer patients examined" (Col. 92, lines 4-6). In the paragraphs discussing these results, Ralph et al. make clear that the experiments were performed using RNA samples prepared from peripheral blood mononuclear cells. This fact is made evident in the first complete paragraph of Col. 94 where they begin the paragraph with the statement: "The relative quantitative RT-PCR studies of Example 5 demonstrated that IL-8 mRNA increases in abundance in the peripheral blood of patients with either metastatic prostate cancer or breast cancer" and end the paragraph with the statement: "As such, by examining the peripheral blood mononuclear cell population, evidence of cancer presence was obtained without requiring any knowledge of its physical location in the body." (Col. 94, lines 8-11 and lines 19-22). In the next paragraph, the authors also explicitly state that: "In this Example, IL-8 mRNA expression was found to be upregulated as much as 15-fold (FIG. 1A and FIG. 2A) in peripheral blood leukocytes of both prostate and breast cancer patients." (Col. 94, lines 29-32). In addition, in Section 4.11.3, the procedure for relative quantitative PCR of IL-8 is presented and the method of RNA preparation from nucleated circulating peripheral blood cells used in this experiment

is described in Section 4.11.1. This procedure specifically refers to "RNA prepared from peripheral blood collected from healthy volunteers, patients with clinically and biopsy confirmed BPH, localized or advanced metastatic prostate cancer, or from patients with advanced metastatic breast cancer" (Col. 67, lines 4-7) and involves the preparation of RNA "from nucleated circulating peripheral blood cells as described in Section 4.9.1" (Col. 67, lines 12-13). In my expert opinion, these specific references to the "peripheral blood mononuclear cell population" and "peripheral blood leukocytes" clearly indicate that the RNA in these experiments was prepared from isolated mononuclear cells.

31. Example 7 concerns the use of relative quantitative RT-PCR for analysis of IL-10 gene expression "using the same protocols as described above for the IL-8 gene". The IL-8 experiments are described under Example 5 and as argued in the preceding paragraph, it is my expert opinion that the RNAs used in Example 5 are derived from the mononuclear cell population.
32. Ralph et al. also describe RNA fingerprinting studies that "more closely resembled the protocol of Welsh et al. (1992)" (Col. 63, lines 15-16) that involves the use of "agarose gels and non-isotopic detection of bands by ethidium bromide staining" (Col. 63, lines 18-19) in Section 4.9.2. In this section they state that: "Total RNAs were isolated from peripheral blood samples as described (Chomczynski & Sacchi, 1987)". The cited reference is the original description of the guanidinium thiocyanate-phenol-chloroform method of RNA isolation in which RNA was prepared from rat mammary tissue, human bone marrow myeloblasts, and rat thyroid FRTL-5 cells. The cited reference also states that: "The method was also used to isolate RNA from cultured cell lines: human and mouse hematopoietic cell lines, breast MCF-7 cells, human normal lymphocytes and bone marrow blasts, and rat thyroid FRTL-5 cells (Chomczynski and Sacchi, Anal. Biochem. 162, 156-159 [1987], p 157, right hand column, 2nd full paragraph, 2nd sentence)." The method described in section 4.9.2 is not specifically referenced in any of the examples presented; however, since all of the relevant examples involve the use of RNA prepared from isolated mononuclear cells and the specific cited reference does not provide any reference to use of unfractionated cells of whole blood, it is my opinion that isolated mononuclear cells were used in the method described in 4.9.2. Further, it is my opinion that this method applies to Example 2 since the method of RNA preparation in Examples 3, 5, and 6 can be traced back to the methods described in section 4.11.1, which refers back to section 4.9.1 and Example 7 uses the method described for IL-8 in Example 5. In my opinion, Example 2 fits most closely with the method presented in 4.9.2 in that RNA fingerprinting is performed by PCR, primed with oligonucleotides of arbitrary sequence (as described by Welsh, 1992) and the "RT-PCR products were electrophoresed through agarose." In this example, Ralph et al. explicitly state that: "For this study, total cell RNA was isolated from buffy coat cells as described above (Col. 79, lines 15-16)." As explained above, buffy coat is another designation used by Ralph et al. for isolated mononuclear cells from peripheral blood. These considerations indicate that the method described in Section 4.9.2 involves the use of the method of Chomczynski and Sacchi to prepare RNA from mononuclear cells in accordance with the procedure outlined in section 4.9.1, even though this is not explicitly stated in Section 4.9.2. The statement in the method that "total RNAs were isolated from peripheral blood samples" cannot be interpreted as designating the isolation of RNA from whole blood, since the authors used similar terminology at various points in the text to refer to RNA prepared from isolated mononuclear cells as argued previously above. Thus, in my opinion, the method described in Section 4.9.2 involves the preparation of RNA from isolated nucleated cells which as described above is used to mean mononuclear cells and there is certainly no explicit description of a method involving the extraction of RNA from whole blood in this section or elsewhere in the text.

fingerprinting results are presented, and therefore this example in WO 98/24935 is not relevant to the issues raised by the examiner.

34. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that wilful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

....., Ph.D.

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EDUCATION

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SUNY Buffalo, Buffalo, NY	B.A.	1975	Biology
Columbia Univ., NY, NY	M.A.	1976	Biology
	M.Phil.	1979	Biology
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RESEARCH AND PROFESSIONAL EXPERIENCE

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1981-83	Post-doctoral Fellow (American Cancer Society)
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PUBLICATIONS

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2. Harpold, M.M., Dobner, P.R., Evans, R.M., and Bancroft, F.C. Nucl. Acids Res. (1978) 5:2039-2053. Construction and identification by positive hybridization-translation of a bacterial plasmid containing a rat growth hormone structural gene sequence.
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Abstract of: Casey et al., 1988. simplified plastic embedding and immunohistologic technique for immunophenotypic analysis of human hematopoietic and lymphoid tissues. Am J Pathol. 131:183-9

Routine fixation and paraffin embedding destroys many hematopoietic and lymphoid differentiation antigens detected by flow cytometry or frozen section immunohistochemistry. On the other hand, morphologic evaluation is difficult in flow cytometric or frozen section studies. A simplified three-step plastic embedding system using acetone-fixed tissues embedded in glycol-methacrylate (GMA) resin has been found to provide both excellent morphologic and antigenic preservation. With our system, a wide variety of antigens are detected in plastic sections without trypsinization or prolonged embedding procedures; **pan-B (CD19, CD22)**, **pan-T (CD7, CD5, CD3, CD2)**, T-subset (CD4, CD8, CD1, CD25) markers as well as surface immunoglobulin and markers for myeloid and mononuclear-phagocyte cells are preserved. In summary, modifications of plastic embedding techniques used in this study simplify the procedure, apparently achieve excellent antigenic preservation, and facilitate evaluation of morphologic details in relation to immunocytochemical markers.

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Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction

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A new method of total RNA isolation by a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture is described. The method provides a pure preparation of undegraded RNA in high yield and can be completed within 4 h. It is particularly useful for processing large numbers of samples and for isolation of RNA from minute quantities of cells or tissue samples. © 1987 Academic Press, Inc.

KEY WORDS: nucleic acids; RNA; messenger RNA; purification; gene expression; human cells.

Guanidinium thiocyanate and chloride are among the most effective protein denaturants (1,2). As a strong inhibitor of ribonucleases, guanidinium chloride was first introduced as a deproteinization agent for isolation of RNA by Cox (3). Since then guanidinium extraction has become the method of choice for RNA purification, replacing phenol extraction. Guanidinium methods have been used successfully by Chirgwin *et al.* (4) to isolate undegraded RNA from ribonuclease-rich tissues like pancreas. Chirgwin's protocol for ultracentrifugation of a guanidinium thiocyanate lysate through a CsCl cushion has become one of the most frequently used for isolation of undegraded RNA. In the present report, a new rapid procedure combining guanidinium thiocyanate and phenol-chloroform extraction is described. A combination of guanidinium and hot phenol for RNA isolation has been reported by Feramisco *et al.* (5). The method we describe differs in that it converts the guanidinium-hot phenol method to a single-step extraction which

allows isolation of RNA in 4 h and provides both high yield and purity of undegraded RNA preparations. By eliminating the ultracentrifugation step of the guanidinium-CsCl method this procedure allows the simultaneous processing of a large number of samples. In addition, this new procedure permits recovery of total RNA from small quantities of tissue or cells making it suitable for gene expression studies for which only a limited quantity of biological material is available.

MATERIALS AND METHODS

Reagents. The denaturing solution (solution D) was 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. To minimize handling of guanidinium thiocyanate (hazardous) a stock solution was prepared as follows: 250 g guanidinium thiocyanate (Fluka) was dissolved in the manufacturer's bottle (without weighing) with 293 ml water, 17.6 ml 0.75 M sodium citrate, pH 7, and 26.4 ml 10% sarcosyl at 65°C. This stock solution can be stored at least 3 months at room temperature. Solution D was prepared by adding 0.36 ml 2-mercaptoethanol/50 ml of stock

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solution. This solution can be stored 1 month at room temperature.

Phenol (nucleic acid grade, Bethesda Research Laboratory) saturated with water was kept at 4°C up to 1 month.

Small-scale RNA preparations were carried out in 4- or 15-ml disposable polypropylene tubes (Falcon, Cat. Nos. 2063, 2059).

Experimental procedure. The acid guanidinium-phenol-chloroform (AGPC)² method was used to isolate RNA from both tissues and cultured cells. The following protocol describes isolation of RNA from 100 mg of rat mammary tissue.

Immediately after removal from the animal, the tissue was minced on ice and homogenized (at room temperature) with 1 ml of solution D in a glass-Teflon homogenizer and subsequently transferred to a 4-ml polypropylene tube. Sequentially, 0.1 ml of 2 M sodium acetate, pH 4, 1 ml of phenol (water saturated), and 0.2 ml of chloroform-isomyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. Samples were centrifuged at 10,000g for 20 min at 4°C. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, and then placed at -20°C for at least 1 h to precipitate RNA. Sedimentation at 10,000g for 20 min was again performed and the resulting RNA pellet was dissolved in 0.3 ml of solution D, transferred into a 1.5-ml Eppendorf tube, and precipitated with 1 vol of isopropanol at -20°C for 1 h. After centrifugation in an Eppendorf centrifuge for 10 min at 4°C the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried (15 min), and dis-

solved in 50 µl 0.5% SDS at 65°C for 10 min. At this point the RNA preparation could be used for poly(A)⁺ selection by oligo(dT) chromatography, Northern blot analysis, and dot blot hybridization. Isopropanol precipitation can be replaced by precipitation with a double volume of ethanol.

The protocol for RNA isolation by the AGPC method is outlined in Table 1. Until the last step, ribonuclease is inhibited by the presence of 4 M guanidinium. Therefore no additional precaution is required to protect RNA from degradation. The final RNA preparation can be dissolved in water or in 1 mM EDTA, pH 8, solution, treated with diethyl pyrocarbonate (DEP) (6). We recommend, however, to use 0.5% SDS (DEP treated) which is a weak inhibitor of ribonuclease and may diminish the effect of accidental contaminations during the further use and storage of RNA samples.

The AGPC method was used for both small scale (3 mg tissue or 10⁶ cells) and large scale (30 g tissue) RNA preparations. The method was also used to isolate RNA from cultured cell lines: human and mouse hematopoietic cell lines, breast MCF-7 cells, human normal lymphocytes, and bone marrow blasts, and rat thyroid FRTL-5 cells. RNA could be isolated from cells grown in suspension (100 µl of solution D per 10⁶ cells) or in monolayer. Cells grown in monolayer were lysed directly in the tissue culture dish by the addition of denaturing solution (1.8 ml of solution D for 10-cm-diameter tissue culture dish).

TABLE 1

AGPC PROTOCOL FOR RNA ISOLATION

1. Extraction	Solution D, 0.2 M sodium acetate, pH 4, phenol, chloroform (1:0.1:1:0.2)
2. Precipitation	1 vol isopropanol
3. Reprecipitation	Solution D, 1 vol isopropanol
4. Wash	75% ethanol
5. Solubilization	0.5% SDS

² Abbreviations used: AGPC, acid guanidinium thiocyanate-phenol-chloroform; SDS, sodium dodecyl sulfate; DEP, diethyl pyrocarbonate.

TABLE 2

COMPARISON OF PARAMETERS OF RNA PREPARATIONS OBTAINED BY THE AGPC METHOD
AND BY THE GUANIDINIUM-CsCl METHOD^a

Method	Ratio ^b (260/280)	DNA ^c	Yield ^d (μ g RNA/mg tissue)	25K casein mRNA ^e (cpm/ μ g RNA)
AGPC	1.85 \pm 0.04	ND	1.76 \pm 0.03	2632 \pm 143
Guanidinium-CsCl	1.75 \pm 0.05	ND	1.52 \pm 0.03	2597 \pm 127

^a Results represent averages of duplicate analyses of three RNA preparations. ND = not detectable.

^b Absorption of RNA preparations was determined at 260 and 280 nm.

^c DNA was quantitated by Burton's reaction (7).

^d Yield was calculated assuming $A_{1\text{ cm}}/0.1\%$ at 260 = 25.

^e 25K casein mRNA was quantitated by hybridization with a ³²P-labeled 25K casein cDNA probe (10).

RESULTS AND DISCUSSION

RNA was prepared from rat mammary tissue by the AGPC extraction and compared with RNA isolated in parallel by ultracentrifugation of guanidinium lysate through a CsCl cushion (4). Data in Table 2 show that RNA isolated by the AGPC method contained less contaminating proteins, as judged by the 260/280 ratio, than RNA isolated by the guanidinium-CsCl method. An additional wash with 75% ethanol of the RNA pellet during the AGPC extraction increased to about 2 the 260/280 ratio of the isolated RNA preparation. DNA was undetectable in both preparations using Burton's method and both had a similar content of 25K casein mRNA. The AGPC extraction, however, resulted in a slightly higher yield of RNA.

Agarose-gel resolution patterns of total RNA isolated from rat mammary gland and rat liver by both the AGPC and the guanidinium-CsCl methods are shown in Fig. 1. Both preparations reveal similar patterns, but liver RNA isolated by the AGPC method contained a greater amount of low-molecular-weight RNA (4–5 S).

The AGPC procedure was used for expression studies of the *ets-2* gene (9) in human bone marrow myeloblasts (low-abundance mRNA), the 25K casein gene (10) in rat mammary gland, and the thyroglobulin gene (11) in FRTL-5 cells (high-abundance mRNAs). AGPC extracted RNA from

human myeloblasts was subjected to oligo(dT) chromatography, and the poly(A)⁺ fraction was analyzed for *ets-2* mRNA content by Northern blot analysis (Fig. 2A). Three transcripts of 4.7, 3.4, and 2.8 kb were detected by an *ets-2* human genomic probe (9) as previously observed using the guanidinium-CsCl method. Total RNA isolated from mammary explants cultured with a lactogenic hormone combination (insulin, prolactin, and hydrocortisone) were probed with 25K casein cDNA and β -actin cDNA (12)

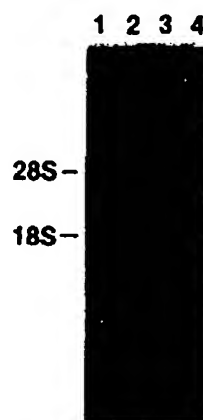


FIG. 1. Electrophoresis of RNA isolated by the AGPC method (lanes 1, 3) and by the guanidinium-CsCl method (lanes 2, 4) from rat mammary gland (lanes 1, 2) and rat liver (lanes 3, 4). RNA preparations (3 μ g) were electrophoresed in formaldehyde-agarose (1%) minigel (8). The gel was washed in water two times for 30 min and stained with ethidium bromide.

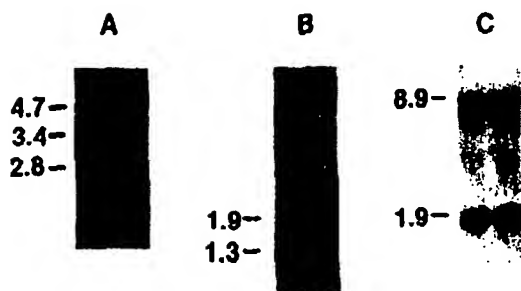


FIG. 2. Northern blot analysis of RNA isolated by the AGPC method. (A) Total RNA from human myeloblasts was chromatographed on oligo(dT) column (15) and 2 μ g of the poly(A)⁺ fraction was analyzed for *ets-2* mRNAs (4.7, 3.4, and 2.8 kb); (B) 1.5 μ g of total RNA from rat mammary explants was analyzed for 25K casein mRNA (1.3 kb) and β -actin mRNA (1.9 kb); (C) 1.6 μ g of total RNA from rat thyroid FRTL-5 cells was analyzed for thyroglobulin mRNA (8.9 kb) and β -actin mRNA. RNA preparations were electrophoresed, transferred to Gene Screen Plus membrane, and autoradiographed as described (9,12).

(Fig. 2B). A 1.3-kb casein mRNA and 1.9-kb β -actin mRNA were detected. Both messengers migrated as distinct bands with no signs of degradation. Thus, despite a high level of ribonuclease activities in rat mammary tissue (13,14), the AGPC method assured high quality of isolated mammary RNA. Finally, undegraded high-molecular-weight thyroglobulin mRNA (8.9 kb) could be visualized in total RNA from rat FRTL-5 cells using a rat thyroglobulin cDNA probe (11) (Fig. 2C).

Presented results show that the AGPC method is a useful alternative to the previously described methods of RNA isolation. The AGPC extraction provides high yield and the extracted RNA is both pure and undegraded. Due to its simplicity and the elimination of ultracentrifugation, the AGPC method allows simultaneous processing of a large number of samples. The method proved to be particularly useful for RNA isolation from as few as 10^6 cells or 3 mg of tissue (human pituitary tumor). The degradation and loss of RNA is minimized by the

limited handling involved in this technique. The AGPC method may therefore be useful for clinical investigations that employ gene expression such as protooncogene expression as a molecular marker of malignancy and tumor progression.

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C

REF 362760
4 mL Draw Capacity
(13 x 100mm tube Size)

REF 362761
8 mL Draw Capacity
(16 x 125mm tube Size)

BD Vacutainer™ CPT™

**Cell Preparation Tube
with Sodium Citrate**

**For the Separation of Mononuclear
Cells from Whole Blood**

STERILE INTERIOR

FOR IN VITRO DIAGNOSTIC USE

Intended Use

The BD Vacutainer™ CPT™ Cell Preparation Tube with Sodium Citrate is an evacuated tube intended for the collection of whole blood and the separation of mononuclear cells. The cell separation medium is comprised of a polyester gel and a density gradient liquid. This configuration permits cell separation during a single centrifugation step. The separated sample can be transported without being removed from the BD Vacutainer™ CPT™ Tube since the gel forms a stable barrier between the cell layers.

Summary and Explanation

Isolation of mononuclear cells from whole blood is a first step for many in vitro assays. One currently accepted technique for mononuclear cell separation, referred to as the Ficoll Hypaque method, employs a liquid density gradient medium of Ficoll 400® and sodium metrizoate or sodium diatrizoate solution ^(1,2,3). The procedure uses anticoagulated blood, collected by routine phlebotomy, which is diluted with a buffered solution, and then carefully layered onto the medium. This preparation is then centrifuged to isolate the mononuclear cells above the medium. The cells are harvested by carefully pipetting them from the liquid interface. The BD Vacutainer™ CPT™ Cell Preparation Tube with Sodium Citrate combines a blood collection tube containing a citrate anticoagulant with a Ficoll Hypaque density fluid and a polyester gel barrier which separates the two liquids. The result is a convenient, single tube system for the collection of whole blood and the separation of mononuclear cells. The BD Vacutainer™ CPT™ Cell Preparation Tube with Sodium Citrate reduces the risk of sample contamination and eliminates the need for additional tubes, pipettes, and reagents. Samples can be transported without removing them from the tube.

TEST PRINCIPLES

The BD Vacutainer™ CPT™ Cell Preparation tube with Sodium Citrate is an evacuated blood collection tube system containing 0.1 M sodium citrate anticoagulant and blood separation media composed of a thixotropic polyester gel and a Ficoll Hypaque solution.

The blood separation media takes advantage of the relatively low density of mononuclear cells to isolate them from whole blood. The separation occurs during centrifugation when the gel portion of the medium moves to form a barrier separating the mononuclear cells and plasma from the denser blood components. The mononuclear cells can be collected by pipetting the cell layer, or the cells can be resuspended into the plasma by gentle inversion to improve cell viability if the sample is to be transported.

REAGENTS, SUPPLIES AND EQUIPMENT

Reagents Provided:

BD Vacutainer™ CPT™ Cell Preparation Tubes with Sodium Citrate.

REF 362760

4 mL Draw Capacity (13 x 100 mm tube Size)
Sterile Tube Interior

Contains:

- 0.45 mL of 0.1 Molar Sodium Citrate Solution (Top Fluid Layer)
- 1.8 gm of Polyester Gel (Middle Layer)
- 1.0 mL of Polysaccharide/Sodium Diatrizoate Solution (Ficoll Hypaque solution, Bottom Fluid Layer)
- Silicone Coated Glass Tube
- Silicone Lubricated Rubber Stopper

REF 362761

8 mL Draw Capacity (16 x 125 mm Tube Size)
Sterile Tube Interior

Contains:

- 1.0 mL of 0.1 Molar Sodium Citrate Solution (Top Fluid Layer)
- 3.0 gm of Polyester Gel (Middle Layer)
- 2.0 mL of Polysaccharide/Sodium Diatrizoate Solution (Ficoll Hypaque solution, Bottom Fluid Layer)
- Silicone Coated Glass Tube
- Silicone Lubricated Rubber Stopper

Reagents Not Provided:

Reagent

- Phosphate Buffered Saline (PBS) without Ca++ or Mg++.

Supplies and Equipment Not Provided:

Specimen Collection

- BD Vacutainer™ Brand Holder and BD Vacutainer™ Brand Needle or BD Vacutainer™ Brand Blood Collection Set.

- Alcohol Swab.
- Dry Sterile Gauze.
- Tourniquet.
- Adhesive Bandage.
- Gloves appropriate for the protection of the person collecting specimen.
- Sharps disposal system.

Specimen Processing

- 15 mL Size Plastic Conical Centrifuge Tubes with Caps.
- Pasteur Pipettes.
- Centrifuge with Swinging Bucket Rotor and Tube Carriers/Adapters for 13 x 100mm and/or 16 x 125mm Tube Size.

NOTE: Centrifuge must be capable of generating at least 1500 RCF at the tube bottom.

- Gloves appropriate for the protection of the person processing specimen.

WARNINGS AND PRECAUTIONS FOR IN VITRO DIAGNOSTIC USE

1. Do not re-use BD Vacutainer™ CPT™ Tubes.
2. Do not use tubes after expiration date printed on the tube label.
3. Do not use tubes if the clear liquid solutions above and below the gel layer become discolored or form precipitates.
4. Do not use tubes for collection of materials to be injected into patients.
5. Since this BD Vacutainer™ CPT™ Tube contains chemical additives, precautions should be taken to prevent possible backflow from the tube during blood drawing (see Prevention of Backflow section).
6. Excessive centrifugation speed (over 2000 RCF) may cause tube breakage, exposure to blood, and possible injury.
7. Remove and reinsert stopper by either gently rocking the stopper from side to side or by grasping with a simultaneous twisting and pulling action. A "thumb roll" procedure for stopper removal is not recommended, as tube breakage and injury may result.
8. **CAUTION:**
 - All glass has the potential for breakage, therefore, precautionary measures should be taken during handling.
 - Handle all biologic samples and blood collection "sharps" (lancets, needles, and blood collection sets) in accordance with the policies and procedures of your facility.

- Obtain appropriate medical attention in the event of any exposure to biologic samples (for example, through a puncture injury) since the samples may transmit HBV (hepatitis), HIV (AIDS), or other infectious diseases.
- Utilize any built-in used needle protector, if the blood collection device provides one. Becton Dickinson does not recommend reshielding used needles, but the policies and procedures of your facility may differ and should always be followed.
- Discard all blood collection "sharps" in biohazard containers approved for their disposal.
- Filling the tubes from a hypodermic syringe while the stopper is in place is not recommended. Forcefully depressing the syringe plunger without removing the stopper can create positive pressure in the tube causing the stopper and specimen to fly out with explosive force.

9. Centrifugation:

CAUTION: If tubes with cracks or chips are used or if excessive speed is used in centrifugation, a tube may break causing the release of sample, droplets, and possibly an aerosol into the centrifuge bowl. The release of these potentially hazardous materials can be mitigated by using specially designed sealed containers in which tubes are held during centrifugation. The use of special containment vessels is not recommended for routine purposes.

Centrifuge carriers and inserts should be of the size specific to the tubes used. Use of carriers too large or too small for the tube may result in breakage. Care should be taken to ensure that tubes are properly seated in the centrifuge cup. Improperly seated tubes may catch on centrifuge head resulting in breakage. Tubes must be balanced in the centrifuge head to minimize the possibility of glass breakage. Always allow centrifuge to come to a complete stop before attempting to remove tubes. When centrifuge head has stopped, open lid and examine for possible broken tubes. If breakage is indicated, use mechanical device such as forceps or hemostat to remove tubes. **Caution: Do not remove broken tubes by hand.**

STORAGE

Store BD Vacutainer™ CPT™ Tubes upright at room temperature (18-25° C). Protect tubes from direct light. Shelf life at 18-25°C is one year from the date of manufacture.

VENIPUNCTURE TECHNIQUE AND SAMPLE COLLECTION Prevention of Backflow

Since this BD Vacutainer™ CPT™ Tube contains chemical additives, it is important to prevent possible backflow from the tube with its attendant possibility

of adverse reactions to the patient. To guard against backflow, the following precautions should be taken when drawing blood into the tube:

1. Keep patient's arm in the downward position during the collection procedure.
2. Hold the tube with the stopper uppermost.
3. Release the tourniquet as soon as the blood starts to flow into the tube, or within 2 minutes of application.
4. Make sure the tube contents do not touch the stopper or the end of the needle during the collection procedure.

Correct Position of Patient's Arm and Tube Assembly to Reduce the Possibility of Backflow

Tourniquet is released as soon as blood starts to flow.

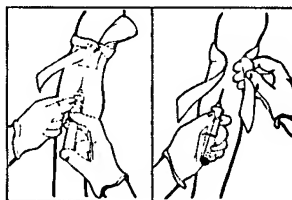


Figure 1

General Instructions

NOTE: Gloves should be worn for venipuncture procedure.

1. Select the tubes appropriate for samples desired.
2. Open needle package but do not remove needle shield. Thread needle onto holder.
3. Insert tube into holder. **LEAVE IN THIS POSITION.**
4. Select site for venipuncture.
5. Apply tourniquet. Prepare venipuncture site with an appropriate antiseptic. **DO NOT PALPATE VENIPUNCTURE AREA AFTER CLEANSING.** Allow site to dry.
6. Remove needle shield. Perform venipuncture **WITH PATIENT'S ARM IN A DOWNWARD POSITION AND TUBE STOPPER UPPERMOST** (see Figure 1). This reduces the risk of backflow of any anticoagulant into the patient's circulation.
7. Push tube onto needle, puncturing diaphragm of stopper.
8. **REMOVE TOURNIQUET AS SOON AS BLOOD APPEARS IN TUBE**, within 2 minutes of venipuncture. **DO NOT ALLOW CONTENTS OF TUBE TO CONTACT THE STOPPER OR THE END OF THE NEEDLE DURING THE PROCEDURE.**

If no blood flows into the tube or if blood ceases to flow before an adequate sample (approximately 3.0 mL as minimum blood volume for 4 mL draw and

approximately 6.0 mL minimum blood volume for 8 mL draw) is collected, the following steps are suggested to complete satisfactory collection:

- a. Confirm correct position of needle cannula in vein.
- b. If a multiple sample needle is being used, remove the tube and place a new tube into the holder.
- c. If the second tube does not draw, remove needle and discard in appropriate disposal device. DO NOT RESHIELD.
Repeat procedure from step 1.

NOTE: When using a blood collection set, a reduced draw of approximately 0.5 mL will occur on the first tube. This reduced draw is due to the trapped air in the blood collection set tubing which enters the first tube.

9. When first tube has filled to its stated volume, remove it from holder.
10. Place succeeding tubes in holder, puncturing diaphragm to initiate flow.
11. While each successive tube is filling invert previous tube 8 to 10 times to mix anticoagulant additive with blood. DO NOT SHAKE. Vigorous mixing can cause hemolysis.
12. As soon as last tube is filled and mixed as above, remove needle from vein. Apply pressure to puncture site with dry, sterile gauze until bleeding stops.
13. Apply bandage, if desired.
14. After venipuncture, the top of the stopper may contain residual blood. Proper precautions should be taken when handling tubes to avoid contact with this blood. Any needle holder that becomes contaminated with blood should be considered hazardous.
15. After collection, dispose of needle using an appropriate disposal device. DO NOT RESHIELD.

PROCEDURE

1. The BD Vacutainer™ CPT™ Tube with Sodium Citrate should be at room temperature (18-25° C) and properly labeled for patient identification.
2. Collect blood into the tube using the standard technique for BD Vacutainer™ Evacuated Blood Collection Tubes (see Venipuncture Technique & Sample Collection section and Prevention of Backflow section).
3. After collection, store tube upright at room temperature until centrifugation. Blood samples should be centrifuged within two hours of blood collection for best results.

4. Centrifuge tube/blood sample at room temperature (18-25° C) in a horizontal rotor (swing-out head) for a minimum of 20 minutes at 1500 to 1800 RCF (Relative Centrifugal Force).

NOTE: Remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times. Also, check to see that the tube is in the proper centrifuge carrier/adaptor.

WARNING: Excessive centrifuge speed (over 2000 RCF) may cause tube breakage and exposure to blood and possible injury. To calculate the correct centrifuge speed for a given RCF, use the following formula:

$$\text{RPM Speed Setting} = \sqrt{\frac{(\text{RCF}) \times (100,000)}{(1.12) \times (r)}}$$

Where r (expressed in centimeters) is the radial distance from the centrifuge center post to the tube bottom, when the tube is in the horizontal position and RCF is the desired centrifugal force, 1500–1800 in this case.

Layering of Formed Elements in the BD Vacutainer™ CPT™ Tube

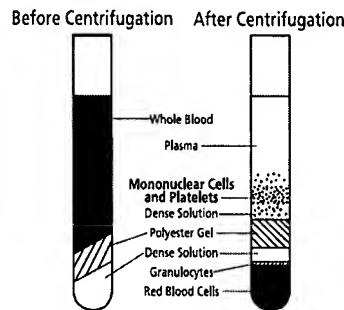


Figure 2

5. After centrifugation, mononuclear cells and platelets will be in a whitish layer just under the plasma layer (see Figure 2). Aspirate approximately half of the plasma without disturbing the cell layer. Collect cell layer with a Pasteur pipette and transfer to a 15 mL size conical centrifuge tube with cap. Collection of cells immediately following centrifugation will yield best results.
6. An alternative procedure for recovering the separated mononuclear cells is to resuspend the cells into the plasma by inverting the unopened BD Vacutainer™ CPT™ Tube gently 5 to 10 times. This is the preferred method for

storing or transporting the separated sample for up to 24 hours after centrifugation. To collect the cells, open the BD Vacutainer™ CPT™ Tube and pipette the entire contents of the tube above the gel into a separate vessel.

Suggested Cell Washing Steps:

1. Add PBS to bring volume to 15 mL. Cap tube. Mix cells by inverting tube 5 times.
2. Centrifuge for 15 minutes at 300 RCF. Aspirate as much supernatant as possible without disturbing cell pellet.
3. Resuspend cell pellet by gently vortexing or tapping tube with index finger.
4. Add PBS to bring volume to 10 mL. Cap tube. Mix cells by inverting tube 5 times.
5. Centrifuge for 10 minutes at 300 RCF. Aspirate as much supernatant as possible without disturbing cell pellet. Resuspend cell pellet in the desired medium for subsequent procedure.

LIMITATIONS

Volume of Blood

The exact quantity of blood drawn will vary with the altitude, ambient temperature, barometric pressure, and venous pressure. The minimum volume of blood that can be processed without significantly affecting the recovery of mononuclear cells is approximately 3.0 mL for 4 mL draw and approximately 6 mL for 8 mL draw. However, hematological parameters such as a low hematocrit or a low mean corpuscular hemoglobin concentration may also adversely affect product performance, with increased red blood cell contamination above gel barrier.

Temperature

Since the principle of separation depends on a density gradient, and the density of the components varies with temperature, the temperature of the system should be controlled between 18-25° C during separation.

Centrifugation

Since the principle of separation depends on the movement of formed elements in the blood through the separation media, the "RCF" should be controlled at 1500 RCF to 1800 RCF. The time of centrifugation should be a minimum of 20 minutes. (As noted in the trouble shooting section, some specimens may require up to 30 minutes for optimal separation.)

Centrifugation of the BD Vacutainer™ CPT™ Tube up to 30 minutes has the effect of reducing red blood cell contamination of the mononuclear cell fraction. Centrifugation beyond 30 minutes has little additional effect. The BD Vacutainer™ CPT™ Tube may be recentrifuged if the mononuclear "band" or layer is not disturbed.

Time

Blood samples should be centrifuged/separated within two hours of blood drawing. Red blood cell contamination in the separated mononuclear cell fraction increases with longer delays in sample separation. Mononuclear cell recovery decreases with increased time delay before centrifugation, falling to approximately 40% mononuclear cell recovery at 24 hours.

Cell Separation

As with other separation media, density gradient separation using BD Vacutainer™ CPT™ Tubes may alter the proportion of some lymphocyte subsets (e.g., T and B cells) from those in unseparated whole blood^(4,5). This alteration is believed to be relatively insignificant in normal cases. However, in cases where the subject is leucopenic or lymphopenic, the selective loss of one subset may alter proportions significantly.

Certain disease states and/or drugs may also alter cell density and therefore affect separation using BD Vacutainer™ CPT™ Tubes⁽⁶⁾.

Microbial Contamination

Microbial contamination of reagents may alter the results obtained on cells separated using BD Vacutainer™ CPT™ Tubes.

Separated Cell Assays

For determinations other than those described in the results section, the user should establish to his or her satisfaction that the values obtained meet his or her criteria for his or her application.

Platelet Contamination

Studies⁽⁷⁾ indicate that mononuclear cell samples separated by the BD Vacutainer™ CPT™ Tube method have approximately 1.3 times the platelet concentration obtained using the Ficoll Hypaque method.

EXPECTED NORMAL DONOR STUDY RESULTS (Using 4 mL Draw Capacity)

Table 1 shows the cell percentages obtained from 45 blood specimens from 32 normal healthy adults using the Ficoll Hypaque and the BD Vacutainer™ CPT™ Tube cell separation methods. Recovery and Purity percentages were determined from the mean of duplicate values obtained using the COULTER Counter® Model S PLUS IV (Coulter Electronics, Inc., Hialeah, FL). Viability percentages were determined by Acridine Orange/Ethidium Bromide staining⁽⁷⁾. Red blood cell percentages were determined by hemocytometer count under a light microscope. Both viability and red blood cell contamination were single determinations for each specimen.

Table 1
Cell Percentages, BD Vacutainer™ CPT™
Citrate Tube Method and
Ficoll Hypaque (FH) Method

PARAMETER		MEAN	SD	CV
Recovery	CPT	71.7	10.5	14.7
	FH	80.9	8.5	10.5
Purity				
Total Mononuclear Cells	CPT	98.0	1.8	1.8
	FH	98.7	0.8	0.9
Lymphocytes	CPT	85.9	4.3	5.0
	FH	87.3	4.0	4.5
Monocytes	CPT	2.1	3.3	27.7
	FH	11.4	3.4	29.7
Viability				
	CPT	99.9	0.3	0.3
	FH	99.7	0.5	0.5
RBC Contamination				
	CPT	14.3	9.4	65.9
	FH	6.8	5.5	81.3
PMN Contamination				
	CPT	2.0	1.8	88.7
	FH	1.2	0.8	68.1

FOOTNOTES:

Recovery – Number of recovered mononuclear cells expressed as a % of the total number of mononuclear cells contained in the original whole blood sample.

Purity – Number of mononuclear cells expressed as a % of the mononuclear cells (lymphocytes and monocytes) in the separated white blood cell fraction.

Viability – Number of mononuclear cells expressed as a % of the total mononuclear cells recovered.

RBC Contamination – Number of red blood cells expressed as a % of the number of separated cells.

Granulocyte (PMN) – Number of granulocytes expressed as a % of the Contamination total number of separated white blood cells.

MEAN – Arithmetic Average

SD – Standard Deviation

CV – Coefficient of Variation (%)

(4 mL Draw Capacity, n = 45)

Mean Blood Draw Volume = 3.83 mL (SD 0.08)

Mean Absolute Mononuclear Cell Count

Recovered = 6.54×10^6 cells per tube (SD 1.99×10^6)

With a Range of 3.36 to 10.54×10^6 cells

(8 mL Draw Capacity, n = 10)

Mean Blood Draw Volume = 7.58 mL (SD 0.08)

Mean Absolute Mononuclear Cell Count

Recovered = 12.72×10^6 cells per tube (SD 4.64×10^6)

With a Range of 7.02 to 21.44×10^6 cells.

PERFORMANCE CHARACTERISTICS

Table 2 shows the reproducibility of separated sample quality using the BD Vacutainer™ CPT™ Tube system which was tested and compared to the Ficoll Hypaque method. Ten samples of one donor's blood were centrifuged and assayed in duplicate for each method. No final washing steps were performed. The samples were resuspended to approximately equal final volumes. Between tube variation was calculated by differencing the mean of the duplicate readings for each tube.

Table 2

Reproducibility Study: Recovery, Purity, Viability, Red Blood Cell (RBC) and Granulocyte (PMN) Contamination** for BD Vacutainer™ CPT™ Citrate Tube Method and Ficoll Hypaque (FH) Method.

PARAMETER		NUMBER	MEAN****	SD	CV%
Recovery*	CPT	9***	70.3	9.7	13.8
	FH	10	82.6	5.5	6.7
Purity*					
Total Mononuclear Cells	CPT	10	98.3	0.6	0.6
	FH	10	99.0	0.3	0.3
Lymphocyte	CPT	10	84.4	1.3	1.5
	FH	10	84.8	0.9	1.1
Monocytes	CPT	10	13.9	1.4	10.4
	FH	10	14.2	0.8	5.9
Viability	CPT	10	100.0	0.0	0.0
	FH	10	99.8	0.4	0.4
RBC Contamination	CPT	10	7.4	1.6	21.7
	FH	10	2.6	1.0	39.0
PMN Contamination	CPT	10	1.7	0.6	34.5
	FH	10	1.0	0.3	30.7

FOOTNOTES:

*Recovery and Purity are based on the Mean of 2 readings.

**Parameters defined in Table 1.

***Sample lost due to spillage, Recovery could not be calculated.

****Using an F-Statistic (2-tailed) at the 5% level, no significant difference was detected between the methods.

MEAN – Arithmetic Mean

SD – Standard Deviation

CV – Coefficient of Variation (%)

N – Number of Tubes

Table 3 shows the cell percentage obtained from 4 groups of 5 patients each, using the Ficoll Hypaque and the BD Vacutainer™ CPT™ Tube cell separation methods. Recovery and purity percentage were determined from the mean of duplicate values obtained using the Coulter® STKR cell counting method. Viability percentages were determined by Acridine Orange/Ethidium Bromide staining⁽⁷⁾.

Table 3

Results from Patient Sample Study

SAMPLE NUMBER	TOTAL MONONUCLEAR RECOVERY (%)		PURITY (%)		VIABILITY (%)	
	CPT	FH	CPT	FH	CPT	FH
Leukemia						
1 CLL	70.5	76.2	98.0	99.4	96.4	97.4
2 CLL	67.8	70.6	99.0	98.5	99.2	98.9
3 CLL	79.7	83.2	97.6	99.5	96.0	97.1
4 CLL	71.4	68.3	99.1	99.3	97.5	98.0
5 CLL	75.6	82.4	99.7	99.7	99.4	100.0
HIV Positive						
6 HIV	62.1	67.3	98.4	97.8	98.5	98.0
7 HIV	59.0	63.7	97.4	98.3	100.0	99.5
8 HIV	68.3	65.6	99.2	96.9	99.2	100.0
9 HIV	63.9	73.0	98.2	97.9	99.3	100.0
10 HIV	70.7	74.8	99.2	98.5	97.2	99.4
Diabetes						
11 DIAB	58.1	64.3	96.7	97.0	99.5	97.8
12 DIAB	66.4	72.3	98.9	99.3	98.9	99.6
13 DIAB	70.2	67.5	97.5	99.0	100.0	99.7
14 DIAB	64.9	70.7	98.5	96.5	99.7	98.2
15 DIAB	62.7	67.7	97.0	98.2	97.8	98.3
Auto Immune						
16 SLE	62.3	64.5	96.4	95.7	97.5	99.0
17 SLE	60.5	63.4	97.0	96.3	99.2	97.3
18 SLE	65.6	62.3	95.7	96.9	96.5	99.0
19 RA	71.5	66.5	96.2	97.2	99.2	100.0
20 RA	69.5	75.6	95.7	97.0	98.9	99.2

FOOTNOTES:

CLL – Chronic Lymphocytic Leukemia

HIV – Human Immunodeficiency Virus Positive

DIAB – Type I Diabetes

SLE – Systemic Lupus Erythematosus

RA – Rheumatoid Arthritis

Recovery, Purity, and Viability Parameters defined in Table 1.

TROUBLESHOOTING

PROBLEM	POSSIBLE	CAUSE SOLUTION
Granulocyte Contamination Greater than 10%.	Centrifuge not at proper speed.	Adjust centrifuge speed to produce 1500-1800 RCF.
	Centrifuge or BD Vacutainer™ CPT™ Tube not at room temperature (18-25°C).	Allow centrifuge and BD Vacutainer™ CPT™ Tube to come to room temperature (18-25°C).
	Delay in centrifugation.	Centrifuge as soon as possible after obtaining blood specimen.
	Abnormal sample with high ratio of granulocytes to mononuclear cells.	Subsequent separation step using standard Ficoll Hypaque method.
Red blood cell contamination.	BD Vacutainer™ CPT™ Tube or centrifuge not at room temperature (18-25°C).	Allow centrifuge or BD Vacutainer™ CPT™ Tube to come to room temperature (18-25°C).
	Centrifugation time too short.	Increase time of centrifugation (up to 30 minutes).
	MCHC below normal ⁽⁸⁾ .	Increase time of centrifugation (up to 30 minutes).
Too few cells.	Leucopenia.	Collect additional BD Vacutainer™ CPT™ specimens as required.
Platelet excess.	High platelet count.	Wash separated cells two times for 15 minutes at 100 RCF.
No defined or distinct mononuclear layer.	Adapter incorrect size.	Use 16 x 125mm centrifuge tube adapter.
	Centrifuge not calibrated correctly.	Have centrifuge calibrated.
	Centrifuge speed too low.	Increase centrifuge speed to produce 1500-1800 RCF.
	Centrifuge time too short.	Increase time of centrifugation (up to 30 minutes).
	Hyperlipemic Sample.	Obtain fasting blood specimen.
No gel movement.	Centrifuge speed too low.	Increase centrifuge speed to produce 1500-1800 RCF.
	Centrifuge temperature less than 18°C.	Increase centrifuge setting to 18-25°C.

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